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Characterization of ATP and P_2 agonists binding to the cardiac plasma membrane P^1,P^4-diadenosine 5′-tetraphosphate receptor

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Abstract

P^1,P^4-Diadenosine 5′-tetraphosphate (Ap_4A) acts as an extracellular modulator through its interaction with purinoceptors. Our laboratory has demonstrated the presence of an Ap_4A receptor in cardiac tissue [1,2]. Due to the rapid hydrolysis of ATP by cardiac membranes the relationship of ATP and Ap_4A binding to purinoceptors on cardiac membranes has not been characterized. In this communication we used two approaches to determine the relationship of ATP to the Ap_4A receptor. Radioligand binding carried out with [α-^{32}P]Ap_4A and adenosine 5′-*O*-{3-thiotriphosphate} ([γ-^{35}S]ATPγS) demonstrates the presence of a single high affinity binding site for Ap_4A and the presence of two binding sites for ATPγS.

The second approach utilized immunoaffinity purified Ap_4A receptor that was shown to be free of ATPase and Ap_4Aase activities. Non-radiolabeled Ap_4A and ATPγS effectively inhibited photocrosslinking of [α-^{32}P]8-N_3Ap_4A to the receptor polypeptide while ATP was a much less effective inhibitor. Furthermore, on plasma membranes [α-^{32}P]8-N_3Ap_4A photocrosslinked to only a 50 kDa polypeptide. These data are consistent with Ap_4A interacting with a homogeneous population of receptors on cardiac plasma membranes but with ATP having a low affinity for the receptor.

Keywords: Ap_4A receptor; ATPγS; ATP; Anti-Ap_4A receptor monoclonal antibody

1. Introduction

In recent years it has become increasingly clear that adenylated dinucleotides act as extracellular modulators of various biological processes. P^1,P^4-Diadenosine 5′-tetraphosphate (Ap_4A) is one of the most abundant and the best characterized adenylated dinucleotide. Ap_4A is co-stored with Ap_3A, Ap_5A Ap_6A and ATP in the dense secretory granules of platelets [3,4]. In chromaffin cells, Ap_4A, Ap_5A, Ap_6A are co-stored with AMP, ADP, ATP and catecholamines [5,6]. It has been estimated that following localized release from platelets and chromaffin cells, Ap_4A could reach physiologically significant concentrations. Furthermore, compared with ATP, Ap_4A has a relatively long half-life in whole blood [7]. Thus, Ap_4A is well suited for a role as an extracellular modulator.

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Among the better characterized targets for extracellular Ap_4A are cells of the vascular system. Ap_4A has been shown to modulate blood vessel tone [8–10], induce the release of nitric oxide from endothelial cells [11], prime respiratory burst and regulate apoptosis in neutrophils [12] and inhibit ADP-induced platelet aggregation [13,14]. Additionally, in other tissues Ap_4A has been shown to promote catecholamine release [15], activate glycogen phosphorylase in hepatocytes [16] and elicit smooth muscle contractions in vas deferens [17] and urinary bladder [18].

The biological effects of Ap_4A on cells have been attributed to its interaction with cell surface receptors where it induces calcium mobilization [19,20]. However, the nature of the receptor(s) in Ap_4A mediated signal transduction remains unclear. In liver plasma membranes Ap_4A appears to bind to a single class of P_{2Y} binding sites [21]. In vas deferens and urinary bladder Ap_4A interacts with P_{2X} receptors [17,18]. However, in brain synaptosomes Ap_4A interacts with two classes of binding sites: a high affinity site which appears to be specific for adenylated dinucleotides (P_{2D}) and a second low affinity site [22].

Our laboratory has demonstrated in partially purified cardiac membrane homogenates that Ap_4A interacts with a P_{2D} receptor and that this receptor undergoes an activation step which enhances only Ap_4A binding [1,2,23]. In addition, investigators have recently demonstrated in cardiac tissue the existence of P_{2X} and P_{2Y} purinoceptors [24,25]. These data suggest that Ap_4A may interact with a heterogeneous population of receptors in cardiac tissue that may consist of specific dipurinoceptors and/or one or more P_2 purinoceptor subtypes. These considerations led us to investigate the relationship of ATP and Ap_4A binding on purified plasma membranes. In this communication we demonstrate that Ap_4A interacts with a homogeneous population of receptors on cardiac plasma membranes while ATP does not bind in appreciable amounts to the Ap_4A receptor.

2. Materials and methods

2.1. Materials

Swiss mice were obtained from Charles River Laboratories. The animals were housed in an animal facility maintained with a photoperiod of 15L:9D and room temperatures of 22–25°C. [α-^{32}P]ATP (3000 Ci/mmol) and [^{32}P]inorganic phosphate (3000 Ci/mmol) were purchased from DuPont New England Nuclear. [γ-^{35}S]ATPγS (1000 Ci/mmol) was purchased from ICN. All nucleotides and goat serum were purchased from Sigma. En^{3}Hance Spray was obtained from DuPont New England Nuclear. Affi-Gel Hz resin, and Kaleidoscope molecular weight markers were purchased from Bio-Rad. DC-Plastikfolien 20×20 cm PEI-Cellulose F plates were purchased from EM Science. All other reagents were of analytical reagent grade or better.

2.2. Synthesis and purification of [α-^{32}P]Ap_4A and [α-^{32}P]8-N_3Ap_4A

[α-^{32}P]Ap_4A and [α-^{32}P]8-N_3Ap_4A were synthesized and purified with yields of about 45% as described [1]. Purity was greater than 95% as determined by thin-layer chromatography and autoradiography.

2.3. Purification of cardiac plasma membranes

Mouse cardiac plasma membranes were purified as described [26]. Briefly, four mouse hearts were suspended in 40 vols. of HEPES buffered saline with glucose and potassium (HBSGK), which contained 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM glucose, 3 mM KCl and 1 mM $CaCl_2$ in addition to 0.1% Triton X-100. The suspended hearts were minced and homogenized with a Dounce homogenizer. After homogenization, the samples were filtered through cheesecloth and centrifuged at 12 000×g for 10 min at 4°C. The supernatant was centrifuged at 100 000×g for 90 min at 4°C and the pellet was resuspended in 0.5 ml of HBSGK buffer containing 0.1% Triton X-100 and 8.5% sucrose (postmitochondrial membrane).

Plasma membranes were separated from sarcoplasmic reticulum by sucrose density centrifugation of the postmitochondrial membrane fraction. Linear sucrose gradients were formed using 19.5 ml of 62.8% sucrose in HBSGK buffer containing 0.1% Triton X-100 and 9.5 ml of 8.5% sucrose HBSGK buffer containing 0.1% Triton X-100. The samples were

centrifuged in a VTi50 rotor at 95 416×*g* for 6 h at 4°C. The plasma membrane fraction was identified using membrane marker enzymes as described [26]. The plasma membranes were stored in aliquots at −80°C for up to 3 months without loss of Ap_4A binding activity.

2.4. Stability of Ap_4A, ATP, ATPγS and α,β-MeATP during binding assays

All experiments were performed in triplicate and repeated three times. Assays were performed in 0.1 ml reaction mixtures. Reaction mixtures contained either 0.9 μM [α-^{32}P]ATP (specific activity 5000–6000 cpm/pmole), 0.5 μM [α-^{32}P]Ap_4A (specific activity 5000–6000 cpm/pmole), 0.6 μM [γ-^{35}S]ATPγS (specific activity 30 000–40 000 cpm/pmole) or 0.9 μM [2,8-^{3}H]α,β-MeATP (specific activity 3000–4000 cpm/pmole) in 67 mM Tris-HCl, pH 7.7 and 0.1 mM $MgCl_2$ (assay buffer). The reactions were initiated by the addition of 2.5 μg of plasma membranes and incubated for various times at 20°C. After incubation, 2 μl of the reaction mixture along with 2 μl non-radiolabeled standards (25 mM of Ap_4A, ATP, ADP, AMP) and [^{32}P]inorganic phosphate (1.0×10^5 cpm) were spotted on DC-Plastikfolien PEI-Cellulose F Plates (TLC plates).

For experiments involving [α-^{32}P]ATP or [α-^{32}P]Ap_4A, TLC plates were developed in 1 M LiCl as described [27]. After development, plates were dried and autoradiography was carried out at −80°C for 4–5 h using Kodak X-OMATAR X-ray film and Cronex Lighting Plus intensifying screens. TLC chromatography with [γ-^{35}S]ATPγS was accomplished using 0.3 M K_2HPO_4, pH 7.0. Autoradiography was carried out at −80°C for 5 h after spraying TLC plates with En^{3}Hance Spray. TLC chromatography with [2,8-^{3}H]α,β-MeATP was carried out in 1 M LiCl as described [27]. Autoradiography was carried out at −80°C for 72–96 h after spraying the TLC plates with En^{3}Hance Spray.

Densitometer tracings of autoradiographs were obtained using a Pharmacia LKB UltraScan XL Enhanced Laser Densitometer connected to an IBM 486 Personal Computer with LKB GelScan XL (version 2.1) software. Percentage of nucleotide hydrolysis was calculated from the relative percentage of total absorbance units ($A_{533}\times mm^2$).

2.5. Radionucleotide binding assays

All experiments were performed in triplicate and repeated at least three times. Ap_4A binding assays were performed as described [28]. Binding assays were performed in 0.1 ml reaction mixtures. Each reaction mixture contained 67 mM Tris-HCl, pH 7.7 and 0.1 mM $MgCl_2$ (assay buffer) and 0.5 μM [^{32}P]Ap_4A (5000–6000 cpm/pmole). Reactions were initiated by the addition of 2.5 μg of plasma membrane and incubated for 15 min at 20°C. Membranes were collected on Whatman R/A glass fiber discs, washed three times with 3 ml portions of 67 mM Tris-HCl, pH 7.7, 0.1 mM $MgCl_2$. The discs were dried and counted in 5 ml of a toluene-based scintillation fluid. Blanks contained all components except membranes.

ATPγS binding was assayed as above except using 0.9 μM [γ-^{35}S]ATPγS (5000–6000 cpm/pmole). Due to non-specific binding of [γ-^{35}S]ATPγS to the glass fiber discs, the glass fiber discs were presoaked in 67 mM Tris-HCl (pH 7.7) and 50 mM sodium pyrophosphate. Membranes were then collected on these discs, washed three times with 3 ml portions of 67 mM Tris-HCl, pH 7.7, 0.1 mM $MgCl_2$ and 10 μM sodium pyrophosphate. The discs were dried and counted in 5 ml of a toluene-based scintillation fluid. Blanks contained all components except membranes.

2.6. Computer analysis of binding data

Saturation binding curves were fitted by a weighted non-linear least square method directly to cpm data and analyzed by the program LIGAND [29]. For displacement experiments, all displacement data were fitted simultaneously by LIGAND. This approach involves a multistep weighted non-linear least squares analysis of single homologous, then heterologous displacement data for each radioligand and finally a simultaneous analysis of all displacement data. This approach yields a more reliable estimation of equilibrium binding parameters for multiple ligands [29]. One, two, and three site models were investigated and compared by the *F*-test with the more complex model accepted only if there was a significant reduction in the sum of squares ($P<0.05$).

2.7. Immunoaffinity purification of the Ap_4A receptor

The Affi-Gel Hz Immunoaffinity Kit (Bio-Rad) was used to generate an anti-Ap_4A receptor monoclonal antibody (mAb TL4) affinity column. Monoclonal antibody TL4 which blocks Ap_4A binding to its cardiac membrane receptor was isolated as described [23]. Monoclonal antibody TL4 (4–5 mg) was exchanged into the coupling buffer (pH 5.5) using an Econ-Pac 10DG desalting column. Monoclonal antibody TL4 carbohydrates were then oxidized in sodium *m*-periodate (20.8 mg/ml) by incubating the sample in the dark for 1 h at room temperature (1:10 $NaIO_4$:mAb TL4 v/v) followed by desalting through an Econ-Pac 10DG desalting column. Oxidized mAb TL4 was coupled to 3 ml of Affi-Gel Hz resin for 24 h at room temperature in coupling buffer. The mAb TL4 coupled resin (Affi-Gel Hz) was washed with 3 bed vols. of PBS (0.145 M NaCl, 38 mM Na_2HPO_4, 11 mM NaH_2PO_4, pH 7.0) containing 0.5 M NaCl and then stored in TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) containing 0.02% NaN_3.

To immunoaffinity purify the Ap_4A receptor the mAb TL4 coupled Affi-Gel Hz resin was blocked by first washing with TBS containing 5% goat serum followed by incubating with TBS containing 20% goat serum overnight at 4°C. Postmitochondrial membrane pellets were resuspended in TBS containing 1% SDS to give a protein concentration of 2 mg/ml. The sample was denatured by boiling for 25 min and then sufficient TBS was added to reduce the SDS to 0.2%. The sample was centrifuged at $16\,000 \times g$ for 30 min and the supernatant incubated overnight at 4°C with mAb TL4 coupled Affi-Gel Hz resin which has been blocked with goat serum. This mixture was packed into a 1.2 cm by 14.5 cm column and washed with 25 ml of TBS. The unbound protein was washed off with 10 mM KH_2PO_4, pH 6.8 until the A_{280} was below 0.01. The bound protein was eluted with 200 mM glycine, pH 2.5 in 2.0 ml fractions and the pH of each fraction was neutralized by the addition of 175 μl of 1.5 mM Tris-HCl, pH 8.8. The protein eluted as a single A_{280} peak and the three tubes with highest absorbance were pooled, dialyzed against three changes of 67 mM Tris-HCl (pH 7.7), concentrated by Centricon-10 (Amicon), and stored at −20°C. A protease inhibitor cocktail containing leupeptin, antipain, chymostatin, pepstatin each at a final concentration of 1 μg/ml and 2 mM phenylmethylsulfonyl fluoride was included in all buffers used in the immunoaffinity purification of the Ap_4A receptor.

2.8. Photolabeling of the immunoaffinity purified receptor

All operations involving 8-azido nucleotides were performed under subdued light. Reaction mixtures contained 5 μg of immunoaffinity purified receptor in 30 μl of assay buffer plus [α-^{32}P]8-N_3Ap_4A (specific activity 1200–1800 cpm/pmole). These reaction mixtures were incubated for 30 min at 20°C in the dark. The samples were irradiated at 4°C using a short-wavelength UV lamp at 254 nm and a dose rate of 300 μW/cm^2 for 60 s. SDS-PAGE and Western transfer were performed as described [1]. Autoradiography was carried out at −80°C for 14–24 h using Kodak X-OMATAR X-ray film and Cronex Lighting Plus intensifying screens. Densitometer analysis of autoradiographs was performed as described above.

2.9. Protein determination

Protein concentrations were determined by the Bradford method [30].

3. Results

3.1. Stability of Ap_4A and ATP during binding assays

Prior to performing binding experiments it is necessary to ensure that the radiolabeled ligands are not hydrolyzed by plasma membrane nucleases under binding conditions. As shown in Fig. 1, [α-^{32}P]ATP is rapidly hydrolyzed with only 64.9% and 33.3% of the radiolabel remaining as ATP after 3 and 15 min of incubation, respectively. In contrast, [α-^{32}P]Ap_4A and [γ-^{35}S]ATPγS were significantly more stable with 80% and 92% of the radiolabel remaining intact after 15 min, respectively (Fig. 1). Furthermore, essentially none of the [^{3}H]α,β-MeATP was hydrolyzed after 60 min (data not shown). Control samples contained all assay components except plasma membranes and

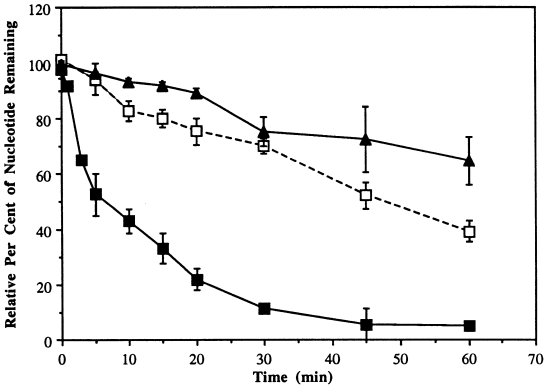

Fig. 1. Time course of nucleotide hydrolysis by cardiac plasma membranes. 2.5 μg of plasma membranes were incubated at 20°C in a final volume of 0.1 ml of binding buffer for the indicated times with 0.9 μM [α-^{32}P]ATP (specific activity 5000–6000 cpm/pmole), 0.5 μM [α-^{32}P]Ap_4A (specific activity 5000–6000 cpm/pmole) or 0.6 μM [γ-^{35}S]ATPγS (specific activity 30 000–40 000 cpm/pmole) for the indicated times. After incubations, 2 μl of the reaction mixture was spotted on TLC plates and separated as described in Section 2. □, Ap_4A; ■, ATP; ▲, ATPγS. The relative percentage was calculated from densitometer tracings of autoradiographs as described in Section 2. The results are expressed as averages of three different experiments in triplicate (nine total data points). Error bars are shown as standard deviations.

were incubated for 60 min prior to TLC. No degradation of the radiolabeled nucleotides was detected in these samples. These data are consistent with purified cardiac plasma membranes containing nucleases which rapidly hydrolyze ATP while hydrolyzing Ap_4A, ATPγS and α,β-MeATP at significantly slower rates.

3.2. Binding characteristics of Ap_4A and ATPγS to cardiac plasma membranes

Due to the rapid hydrolysis of ATP, this ligand was not used in binding studies. The binding of 0.5 μM [α-^{32}P]Ap_4A to cardiac membranes was linear with protein concentrations from 1.0 μg to 4.0 μg while the binding of 0.9 μM [γ-^{35}S]ATPγS was linear with protein concentrations from 1.0 μg to 25.0 μg (data not shown). Ap_4A binding to 2.5 μg of plasma membranes reached a plateau after 12 min. Longer incubations up to 30 min did not significantly increase the maximal amount of [α-^{32}P]Ap_4A bound (data not shown). [γ-^{35}S]ATPγS binding to 2.5 μg cardiac plasma membranes reached a plateau after 10 min. Incubations up to 30 min did not significantly increase the maximal amount of [γ-^{35}S]ATPγS bound (data not shown). These results demonstrated that binding equilibrium was attained within a time where hydrolysis would not be a factor. All subsequent Ap_4A and ATPγS binding experiments were performed using 2.5 μg of plasma membrane for 15 min at 20°C.

3.3. Specificity of Ap_4A and ATPγS binding to cardiac plasma membranes

In an attempt to determine binding parameters for Ap_4A and ATPγS, binding experiments were performed using a variable amount of either [α-^{32}P]Ap_4A or [γ-^{35}S]ATPγS and a fixed amount of plasma membrane (Fig. 2). [α-^{32}P]Ap_4A binding plateaued at 0.25 μM while [γ-^{35}S]ATPγS plateaued at 0.6 μM. Binding data are displayed in the Scatchard coordinate system and indicated a linear relationship for Ap_4A (Fig. 3A) which suggests that there is a single binding site for Ap_4A. Data analysis with the program LIGAND is consistent with one class of high affinity binding sites for Ap_4A with an apparent receptor density (B_{max}) of 66 pmoles mg^{-1} of membrane protein and an apparent K_d value of 0.05 μM (Table 1). On the other hand, Scatchard analysis

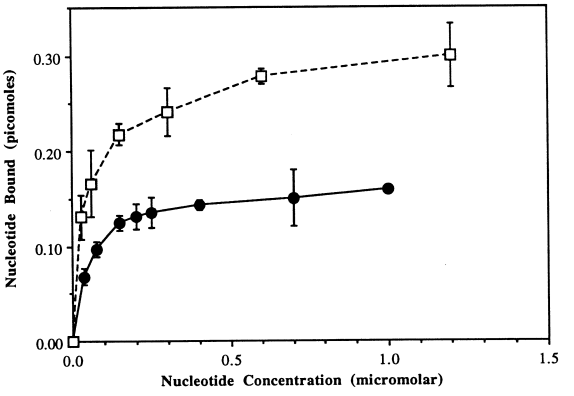

Fig. 2. [α-^{32}P]Ap_4A and [γ-^{35}S]ATPγS binding to cardiac plasma membranes. The binding assay is described in the text. Varying concentrations of [α-^{32}P]Ap_4A (specific activity 6000–9000 cpm/pmole) or [γ-^{35}S]ATPγS (specific activity 6000–9000 cpm/pmole) were incubated with 2.5 μg of plasma membranes for 15 min at 20°C. ●, Ap_4A; □, ATPγS. The results are expressed as averages of three different experiments performed in triplicate (nine total data points). Error bars are shown as standard deviations.

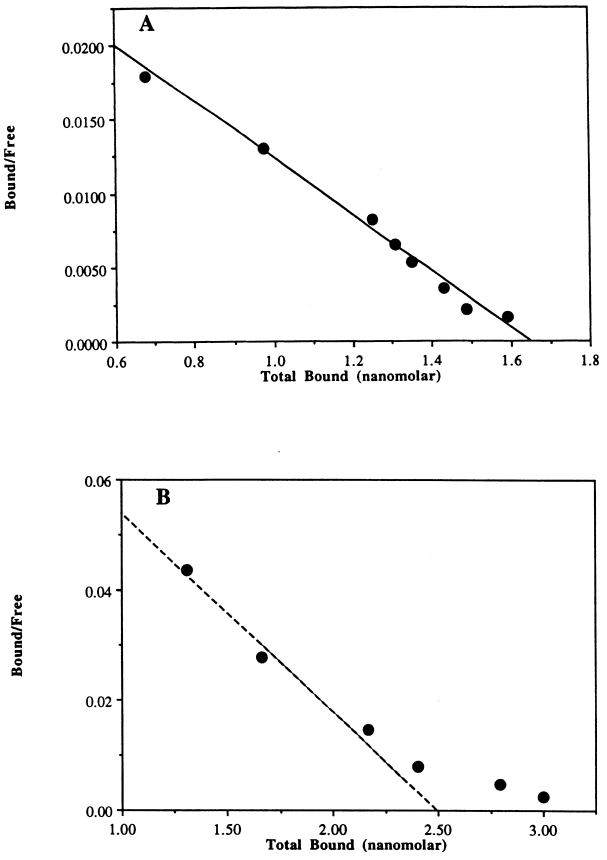

Fig. 3. Scatchard analysis of [α-^{32}P]Ap_4A and [γ-^{35}S]ATPγS binding to cardiac plasma membranes. Experiments were performed as described in the legend of Fig. 2. The data were analyzed by LIGAND [29] and are displayed as Scatchard plots. (A) Scatchard analysis of Ap_4A binding data. The solid line represents the Scatchard parameters generated from linear regression analysis. (B) Scatchard analysis of ATPγS binding data. The dashed line represents the line generated by the apparent K_d derived from LIGAND after subtracting non-specific binding.

showed a non-linear relationship for ATPγS (Fig. 3B) which suggests there are either two classes of binding sites for ATPγS or there are both specific high affinity binding sites and non-specific binding sites. When using the LIGAND program the analysis is dominated by high affinity interactions. Due to the weighted analysis, the apparent ATPγS K_d value has an upper limit value of 0.03 μM for the high affinity site and an apparent B_{max} of 99 pmoles mg^{-1} of membrane protein (Table 1).

Equilibrium binding parameters were also determined by performing homologous and heterologous competition studies of [α-^{32}P]Ap_4A and [γ-^{35}S]ATPγS binding using non-radiolabeled Ap_4A, ATPγS, α,β-MeATP and adenosine. The ability of non-radiolabeled Ap_4A, ATPγS and α,β-MeATP to inhibit [α-^{32}P]Ap_4A binding is presented in Fig. 4A. Variable amounts of non-radiolabeled nucleotides and a fixed amount of [α-^{32}P]Ap_4A were incubated with plasma membranes for 15 min at 20°C. As expected, non-radiolabeled Ap_4A effectively competes with [α-^{32}P]Ap_4A for binding sites. Non-radiolabeled ATPγS competed as effectively as did non-radiolabeled Ap_4A. However, the P_{2X} agonist, α,β-MeATP, was not an effective competitor of [α-^{32}P]Ap_4A binding by at least two orders of magnitude.

The ability of non-radiolabeled Ap_4A, ATPγS, α,β-MeATP to compete for [γ-^{35}S]ATPγS binding is presented in Fig. 4B. Variable amounts of non-radiolabeled nucleotides and a fixed amount of [γ-^{35}S]ATPγS were incubated with plasma membranes for 15 min at 20°C. Non-radiolabeled ATPγS, as expected, effectively competes for [γ-^{35}S]ATPγS binding sites. However, non-radiolabeled Ap_4A was not an effective competitor of [γ-^{35}S]ATPγS binding. In fact 1 mM Ap_4A inhibits only 60% of [γ-^{35}S]ATPγS binding. This is in contrast to the reverse experiment in which non-radiolabeled ATPγS effectively inhibited [α-^{32}P]Ap_4A binding (Fig. 4A). α,β-MeATP was also not an effective competitor of [γ-^{35}S]ATPγS binding with only 60% inhibition at a concentration of 1 mM. These data are consistent with the presence of a second class of low affinity binding sites for ATPγS which is not effectively competed for by Ap_4A or α,β-MeATP.

The P_{2X} purinoceptor agonist α,β-MeATP did not effectively compete with either [α-^{32}P]Ap_4A (Fig. 4A) or [γ-^{35}S]ATPγS (Fig. 4B) binding. In addition adenosine did not compete with either [α-^{32}P]Ap_4A or [γ-^{35}S]ATPγS binding at concentrations up to 10 mM (data not shown). These data are consistent with neither the high nor low affinity binding sites being a P_{2X} or P_1 receptor.

Computer analyses of all homologous and heterologous displacement studies were fitted simultaneously with LIGAND and predicted the presence of one high affinity binding site for both Ap_4A and ATPγS and an additional low affinity binding site for ATPγS. At the high affinity binding site the apparent K_d values for Ap_4A and ATPγS are 0.08 μM and 0.04 μM, respectively (Table 1). Non-specific

Table 1
K_d values for nucleotide binding to cardiac plasma membranes

Nucleotide	Scatchard analysis[a]	Competition analysis[b]	
	K_d high affinity site (μM)	K_d high affinity site (μM)	K_d low affinity site (μM)
Ap_4A	0.05	0.08	–
ATPγS	0.03[c]	0.04	1.0
α,β-MeATP	nd[d]	3.0	–

[a]2.5 μg of plasma membranes were incubated with varying amounts of either [α-^{32}P]Ap_4A (specific activity 6000–9000 cpm/pmole) or [γ-^{35}S]ATPγS (specific activity 6000–9000 cpm/pmole) in a final volume of 0.1 ml as described in the legend of Fig. 2. Saturation binding curves were fitted directly to raw cpm data and analyzed by the program LIGAND as described in the text.
[b]2.5 μg of plasma membranes were incubated with either 0.5 μM [α-^{32}P]Ap_4A (specific activity 6000–9000 cpm/pmole) or 0.9 μM [γ-^{35}S]ATPγS (specific activity 6000–9000 cpm/pmole) and varying amounts of competing non-radiolabeled ligand (0.4 nM–1.0 mM) in a final volume of 0.1 ml as described in the legend of Fig. 4. Data from all homologous and heterologous displacement experiments were simultaneously fitted and analyzed by the program LIGAND as described in the text.
[c]Upper limit K_d value for the high affinity site.
[d]Equilibrium binding parameters were not determined by this method.

binding ratios of 0.001 and 0.0007 were calculated for Ap_4A and ATPγS, respectively, indicating that less than 0.1% of the total high affinity binding was due to non-specific interactions. The apparent K_d value for the low affinity ATPγS site is 1.0 μM (Table 1); however, the non-specific binding ratio of 0.012 is relatively high compared to those for the high affinity site. These data are consistent with Ap_4A and ATPγS binding to the same high affinity site on cardiac plasma membranes.

3.4. *Identification of the Ap_4A receptor by photocrosslinking [α-^{32}P]8-N_3Ap_4A to plasma membranes*

To identify the Ap_4A receptor, 5 μg of plasma membranes were incubated with [α-^{32}P]8-N_3Ap_4A for 30 min at 20°C prior to UV crosslinking. SDS-PAGE followed by Western transfer and autoradiography revealed [α-^{32}P]8-N_3Ap_4A photolabeling of a 50 kDa and a smaller amount of a 30 kDa polypeptide (Fig. 5). Since we have previously shown that the 30 kDa polypeptide is a consequence of artifactual receptor proteolysis [26], these data are consistent with Ap_4A binding to a 50 kDa polypeptide on purified cardiac plasma membranes.

The hydrolysis of ATP by cardiac plasma membranes prevented the use of ATP in radioligand binding studies. In an attempt to determine the interactions of ATP with the cardiac Ap_4A receptor, studies using immunoaffinity purified Ap_4A receptor were performed. The Ap_4A receptor polypeptide was immunoaffinity purified with an anti-Ap_4A receptor mAb (mAb TL 4) that we have raised against the mouse cardiac receptor [23]. The immunoaffinity purified receptor was photolabeled with varying concentrations of [α-^{32}P]8-N_3Ap_4A, followed by SDS-PAGE, Western transfer and autoradiography. As shown in Fig. 6, photolabeling with [α-^{32}P]8-N_3Ap_4A was linear for concentrations of 5–20 μM and reached a plateau at 20 μM.

The ability of non-radiolabeled Ap_4A, ATPγS and ATP to inhibit [α-^{32}P]8-N_3Ap_4A photocrosslinking to purified polypeptide fractions is shown in Fig. 7. Ligand specificity of the Ap_4A receptor was determined by incubating variable amounts of non-radiolabeled nucleotides and a fixed amount of [α-^{32}P]8-N_3Ap_4A prior to UV crosslinking, SDS-PAGE, Western transfer and autoradiography. As expected both Ap_4A and ATPγS effectively inhibit [α-^{32}P]8-N_3Ap_4A crosslinking to the receptor polypeptides. However, ATP is significantly less effective at competing for [α-^{32}P]8-N_3Ap_4A (Fig. 7). The estimated IC_{50} values for ATPγS, Ap_4A and ATP are 56 μM, 158 μM and 2500 μM, respectively. Higher concentrations of Ap_4A and ATPγS were required for competing with [α-^{32}P]8-N_3Ap_4A photocrosslinking (Fig. 7) than with [^{32}P]Ap_4A binding to plasma membranes (Fig. 4). The higher concentration of non-photoactive ligand that is required to inhibit photolabeling can be explained on the basis of reversible binding of the non-photoactive ligand and the cova-

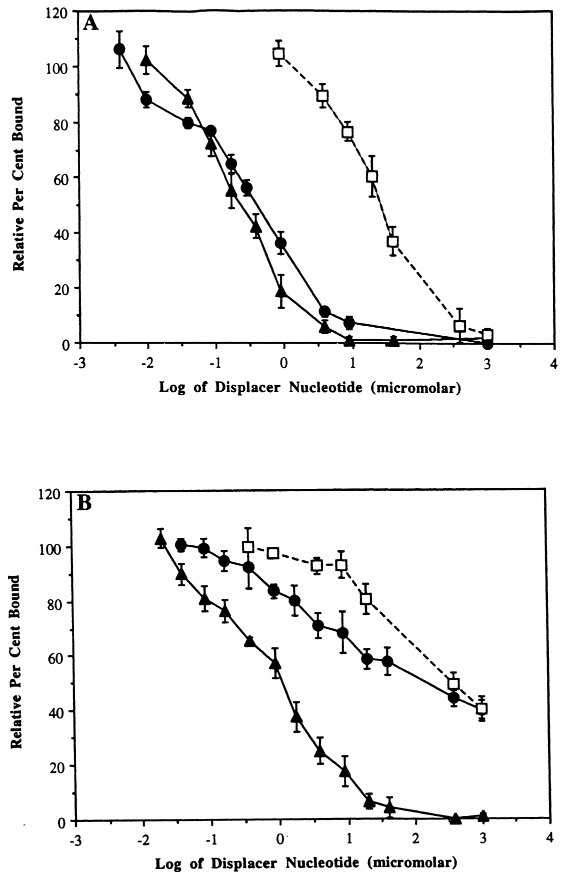

Fig. 4. Inhibition of [α-^{32}P]Ap_4A and [γ-^{35}S]ATPγS binding to cardiac plasma membranes by unlabeled Ap_4A, ATPγS and α,β-MeATP. The binding assay is described in the text. (A) 2.5 μg of plasma membranes were incubated with 0.5 μM [α-^{32}P]Ap_4A (specific activity 6000–9000 cpm/pmole) and varying concentrations of non-radiolabeled ligand for 15 min at 20°C. ●, Ap_4A; ▲, ATPγS; □, α,β-MeATP. (B) 2.5 μg of plasma membranes were incubated with 0.9 μM [γ-^{35}S]ATPγS (specific activity 6000–9000 cpm/pmole) and varying concentrations of non-radiolabeled nucleotide for 15 min at 20°C. ●, Ap_4A; ▲, ATPγS; □, α,β-MeATP. Results are expressed as averages of at least six experiments in duplicate (12 total data points). Error bars are shown as standard deviations.

lent binding of the photoactive ligand [31]. In addition, similar observations have been made by other investigators who found that excess cAMP and Ap_4A were required to inhibit photoincorporation of 8-N_3-cAMP and 8-N_3Ap_4A, respectively [31,32].

To ensure that the immunoaffinity purified Ap_4A receptor did not contain Ap_4A hydrolyases or ATPases, immunoaffinity purified receptor was incubated with [^{32}P]Ap_4A, [γ-^{32}P]ATP or [γ-^{35}S]ATPγS for 30 min at 20°C. Aliquots were subjected to TLC followed by autoradiography. Greater than 90% of radiolabeled [^{32}P]Ap_4A, [γ-^{32}P]ATP and [γ-^{35}S]ATPγS co-migrates with non-radiolabeled Ap_4A, ATP and ATPγS, respectively (data not shown). Furthermore, the design of [α-^{32}P]8-N_3Ap_4A is such that no likely degradation products can contain both radiolabel and photoactive azido group, thus ensuring that any labeling is due to the intact molecule. These data are consistent with Ap_4A and ATPγS specifically interacting with the cardiac plasma membrane Ap_4A receptor but with ATP having a low affinity for this receptor.

4. Discussion

In different tissues and cell types Ap_4A has been shown to interact with P_{2Y}, P_{2X}, as well as P_{2D} purinoceptors [1,2,17,18,21,22,24,25]. Furthermore, evidence from physiological and molecular studies suggests that a heterogeneous population of purinoceptors exists within cardiac tissue [24,25]. However, the relationship of Ap_4A to the various purinoceptors in cardiac plasma membranes is not clear. The work presented in this communication is consistent with Ap_4A and ATPγS interacting with a homogeneous population of receptors on cardiac plasma membranes and that ATP has a low affinity for the Ap_4A receptor.

The presence of ectonucleotidases that rapidly hy-

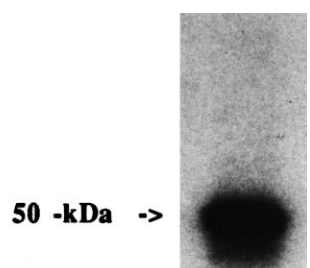

Fig. 5. SDS-PAGE and autoradiography of [α-^{32}P]8-N_3Ap_4A photolabeled cardiac plasma membrane. Plasma membrane was isolated as described in the text. 5.0 μg of plasma membrane were incubated in 30 μl of assay buffer with 1.2 nM [α-^{32}P]8-N_3Ap_4A (specific activity 4.8×10^7 cpm/pmole) for 30 min at 20°C prior to UV crosslinking and SDS-PAGE as described in the text. Molecular weights are denoted by the arrows.

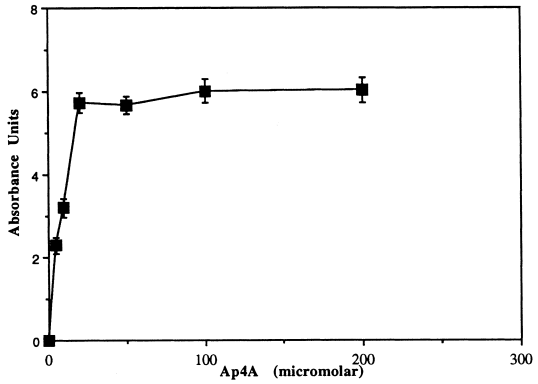

Fig. 6. SDS-PAGE and autoradiography of [α-^{32}P]8-N_3Ap_4A photolabeled immunoaffinity purified Ap_4A receptor 50 kDa polypeptide. 5.0 μg of immunoaffinity purified receptor were incubated in 30 μl of assay buffer with varying amounts of [α-^{32}P]8-N_3Ap_4A (specific activity 1200–1800 cpm/pmole) for 30 min at 20°C prior to UV crosslinking and SDS-PAGE as described in the text. Photolabeling was detected by autoradiography and quantified by densitometry as described in the text. Absorbance units are equal to $A_{533} \times mm^2$. The results are expressed as an average of three different experiments in triplicate (nine total data points). Error bars are shown as standard deviations.

drolyze ATP and adenylated dinucleotides may preclude direct investigations into the action of these nucleotides on many tissues and cells. Hulme and Birdsall have suggested that greater than 75% of the radioligand must remain intact to obtain reliable equilibrium binding parameters [33]. Optimal binding conditions required a 15 min incubation at 20°C to obtain equilibrium. Following a 15 min incubation, 80.2%, 91.7%, 100% and 33.3% of the radiolabeled Ap_4A, ATPγS, α,β-MeATP and ATP migrated with control standards, respectively (Fig. 1). These data demonstrate that Ap_4A, ATPγS, α,β-MeATP meet the Hulme and Birdsall criteria, while using ATP in receptor-ligand studies would be biased by unknown concentrations of hydrolytic products.

Considering the rapid hydrolysis of ATP by cardiac plasma membranes two synthetic ATP analogs were selected for use in radioligand binding studies. We chose ATPγS, a potent P_{2Y} agonist [34], and α,β-MeATP, a potent P_{2X} agonist [18], to investigate mononucleotide interactions with the Ap_4A receptor on cardiac plasma membranes. Homologous and heterologous competition studies carried out with [α-^{32}P]Ap_4A and [γ-^{35}S]ATPγS are consistent with Ap_4A interacting with only a single class of high affinity binding site while ATPγS interacts with this same class of high affinity sites and a second low affinity site (Fig. 4 and Table 1). Apparent K_d values for the high affinity site obtained from competition studies for Ap_4A and ATPγS are in good agreement with the values obtained from Scatchard analysis (Table 1). Computer curve-fitting of both competition and Scatchard analysis is consistent with Ap_4A binding to a homogeneous population of receptors on cardiac plasma membranes.

The use of multiple radioligands and two independent methods for determining equilibrium binding parameters not only strengthens the notion of a single high affinity Ap_4A receptor but also allows for the observation of a two binding site model for ATPγS. Although Ap_4A and ATPγS both effectively competed for [α-^{32}P]Ap_4A binding, Ap_4A only competed with 60% of the total [γ-^{35}S]ATPγS binding (Fig. 4B). These data are consistent with the presence of at least two binding sites for ATPγS on cardiac plasma membranes.

The apparent K_d values of 0.03 and 0.04 μM for ATPγS (Table 1) are similar to the values reported for ATPγS binding to P_{2Y} receptors on endothelial

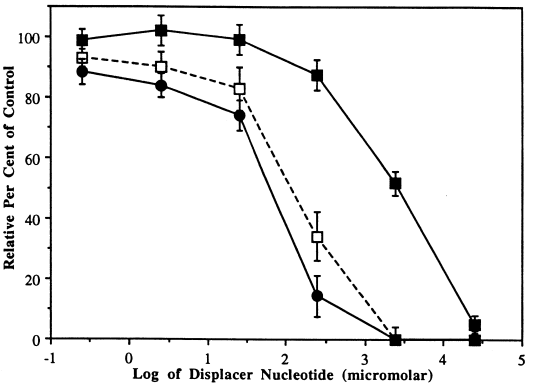

Fig. 7. Inhibition of [α-^{32}P]8-N_3Ap_4A binding to the immunoaffinity purified Ap_4A receptor 50 kDa polypeptide by unlabeled Ap_4A, ATPγS and ATP. 5.0 μg of immunoaffinity purified receptor were incubated in 30 μl of assay buffer with 25 μM [α-^{32}P]8-N_3Ap_4A (specific activity 1200–1800 cpm/pmole) and various concentrations of non-radiolabeled Ap_4A, ATPγS, or ATP for 30 min at 20°C prior to UV crosslinking and SDS-PAGE as described in the text. Photolabeling was detected by autoradiography and quantified by densitometry as described in the text. Absorbance units are equal to $A_{533} \times mm^2$. □, Ap_4A; ●, ATPγS; ■, ATP. The results are expressed as an average of three different experiments in triplicate (nine total data points). Error bars are shown as standard deviations.

membranes (0.01 μM) [35] and for ATPαS binding to P_{2Y} receptors on liver plasma membranes (0.19 μM) [36,37]. Ap_4A binds to a single class of P_{2Y} binding sites on liver plasma membranes with an apparent K_d value (1.76 μM) [21] that is about 33-fold higher than the value we obtained from cardiac plasma membranes (Table 1). On the other hand, Ap_4A appears to bind to two classes of binding sites on brain synaptosomes and chromaffin cells with apparent K_d values of 0.1 nM and 0.08 nM for the high affinity sites and apparent K_i values of 0.57 μM and 0.13 μM for the low affinity sites, respectively [22,38]. These data suggest that there may be heterogeneity of Ap_4A binding sites in the various tissues.

Competition studies indicate that Ap_4A and ATPγS have similar binding affinities for the high affinity site while α,β-MeATP and adenosine were ineffective competitors of either [α-^{32}P]Ap_4A or [γ-^{35}S]ATPγS. Other investigators have demonstrated that ATPαS and α,β-MeATP bind to different P_{2Y} and P_{2X} purinoceptors on brain synaptosomal membranes, respectively [39]. These data are consistent with the high affinity Ap_4A binding site on cardiac plasma membranes not being a P_{2X} or P_1 receptor.

Our apparent B_{max} value of 66 pmol mg^{-1} for the Ap_4A receptor on cardiac plasma membranes is within the range of receptor densities observed for Ap_4A in liver plasma membranes (47 pmol mg^{-1}) [21]. However, the Ap_4A cardiac plasma membrane receptor density is more than three orders of magnitude greater than the B_{max} values for the high affinity Ap_4A binding site on brain synaptosomes (16 fmoles mg^{-1}) [22]. These data suggest that there are variations of Ap_4A receptor densities in different tissues.

On purified cardiac plasma membranes [α-^{32}P]8-N_3Ap_4A only photolabeled a 50 and a 30 kDa polypeptide (Fig. 5). Since we have previously shown that the 30 kDa polypeptide is a consequence of artifactual receptor proteolysis [26], these data are consistent with Ap_4A binding to a 50 kDa polypeptide on purified cardiac plasma membranes. However, we have also demonstrated that on isolated cardiac myocytes [α-^{32}P]8-N_3Ap_4A photolabeled only a 42 kDa polypeptide [26]. We have also previously demonstrated that the Ap_4A receptor undergoes at least two proteolytic processing steps, one of which is carried out by a serine protease [1,2,23] and that this serine protease is required for receptor activation. Since the serine protease is separated from the plasma membrane during plasma membrane purification (unpublished observation), this suggests that the Ap_4A receptor on purified cardiac plasma membranes has not been activated and that the 50 kDa polypeptide represents the Ap_4A receptor prior to processing. The apparent K_d value for Ap_4A on cardiac plasma membranes (Table 1) is 4-fold higher than the value obtained for activated partially purified cardiac membrane homogenates [2]. Although unactivated, the higher purification of plasma membranes allows for a more accurate estimation of binding affinity than studies with partially purified membrane homogenates. This is evidenced by the 8-fold lower K_d value obtained for Ap_4A with purified plasma membranes than the K_d value (0.65 μM) obtained for unactivated partially purified membrane homogenates [2]. Nevertheless, these data are consistent with Ap_4A interacting with a single 50 kDa polypeptide on cardiac plasma membranes.

A number of P_{2Y} agonists have been shown to have varying effects on Ap_4A binding to membrane receptors. The P_{2Y} agonist ADPβS is an effective competitor of radiolabeled Ap_4A binding to brain synaptosome [22] and chromaffin cell P_{2D} purinoceptors [38]. ATPγS is nearly as effective as Ap_4A at activating cloned P_{2U} receptors expressed in astrocytoma cells [40]. On the other hand Ap_4A does not compete with the P_{2Y} agonist ATPαS binding to brain synaptosomes [39] or liver P_{2Y} receptors [36]. These observations suggest that classification of the Ap_4A receptor as a P_{2D} purinoceptor should not be accomplished by only using synthetic ATP analogs because their specificity may be cell and tissue specific. In addition, due to the presence of enzymes on purified cardiac plasma membranes that rapidly hydrolyze ATP (Fig. 1), direct investigations of the action of ATP is precluded. Consequently, a second approach was required to assess the relationship of ATP to the Ap_4A receptor. This was accomplished by photoaffinity labeling studies of the Ap_4A receptor immunoaffinity purified using mAb TL4. Monoclonal antibody TL4 has been shown to interact with part of the Ap_4A binding domain [41]. Both non-labeled ATPγS and Ap_4A were effective competitors of [α-^{32}P]8-N_3Ap_4A with estimated IC_{50} values of 56 and 158 μM, respectively. In contrast, non-labeled

ATP was not an effective inhibitor of [α-^{32}P]8-N_3Ap_4A (estimated IC_{50} of 2500 μM) (Fig. 7). These data demonstrate that ATP does not bind in appreciable amounts to the Ap_4A receptor.

The data presented in this communication are consistent with Ap_4A interacting with a homogeneous population of receptors on cardiac plasma membranes and with ATP having a low affinity for this receptor. These data also support our previous observations that the cardiac Ap_4A receptor is a unique dipurinoceptor which is highly specific for adenylated dinucleotides [1,2]. However, final placement of the cardiac Ap_4A receptor as a distinct P_2 purinoceptor subclass must await the isolation and sequencing of Ap_4A receptor full length cDNA clones.

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